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Mechanisms of Quenching of Alexa Fluorophores by Natural Amino Acids

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Quenching of fluorophores by the same proteins to which they are linked is a phenomenon that is neither well-known nor well-characterized. Fluorescence spectroscopy has become an important tool for studying protein conformations and protein-protein interactions. Extrinsic fluorescent molecules, with reactive side groups, are covalently attached to specific labeling sites within the proteins of interest. Changes in fluorescence intensity or lifetime report on changes in the fluorophore's local environment. Mechanisms such as Förster Resonance Energy Transfer (FRET) also allow for precise measurement of the distances between acceptor and donor fluorophores attached to various parts of a protein¹.

Although proteins are usually assayed after labeling to ensure that the attached fluorophore(s) do not affect their intrinsic structure and function, the effect of the target protein itself on the attached fluorophore is not often considered when making quantitative measurements of fluorescence intensity. Here, we elucidate the quenching effects that the protein itself can have on the fluorophore attached to it, using members of the Alexa Fluor series of dyes as our model. Alexa Fluor dyes from Invitrogen™ are often used to label proteins, because they are more stable to photobleaching or conversions to the triplet states². Their high quantum efficiencies make them suitable for single molecule studies where brightness is an important factor. Pairs like Alexa dyes 488/555 and Alexa dyes 488/594 are often used as conformational reporters in FRET experiments.

Spacers, such as a short saturated carbon chain, can be added between the fluorophore and the attachment site to mitigate unwanted interactions, but their flexibility still allows for contact with nearby amino acids. Indeed, fluorescence quenching as a result of contact with amino acids within the same molecule has been observed, and exploited to report on changing conformational states^{3,4} or intramolecular dynamics^{5–7}. While the specific mechanism of quenching is unknown, it has been attributed to photoinduced electron transfer (PET), wherein a molecule (donor) excited by light transfers its excited state electron to another molecule (acceptor) when they are in close van der Waals contact.

In this communication, we identify amino acids that quench the fluorescence of Alexa dyes 488 (AL488), 555 (AL555) and 594 (AL594). We identify four quenchers of AL488 –

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Supporting Information Available: Experimental details for fluorescence and electrochemical experiments.

Tryptophan (Trp), Tyrosine (Tyr), Histidine (His) and Methionine (Met). We observe a combination of static and collisional mechanisms in the quenching of AL488.

Fluorescence emission scans and two-photon excitation lifetime measurements of AL488, AL555 and AL594 hydrazides were performed both in the presence and absence of the 20 naturally occurring L-amino acids (experimental details can be found in Supporting Information). We identified Trp as a common quencher for all three fluorophores. We observed that Tyr, His and Met also quench AL488 fluorescence. While Trp and Tyr are known to quench certain fluorophores^{5,8}, the inclusion of His and Met was surprising. Fluorescence quenching can be broadly classified as arising from dynamic or static mechanisms⁹. (Theoretical background can be found in Supporting Information.) Comparing the fluorescence intensity Stern-Volmer plots of all four amino acids (Fig. 1), we observed that Trp and Tyr are strong quenchers of AL488 fluorescence, while His and Met are weak quenchers. The difference between the slopes of the lifetime and the intensity measurements indicate that quenching of AL488 by Trp has both static and dynamic components (Fig. 2a), with a dynamic quenching rate of $3.5 \times 10^9 \text{M}^{-1} \text{s}^{-1}$. The predicted collision rate between AL488 and Trp in solution as calculated from the Smulochowski equation is $9.3 \times 10^9 \text{M}^{-1} \text{s}^{-1}$. The fraction of total collisions that result in fluorescence quenching is 0.379 ± 0.006 . This value may indicate a dependence on the relative orientations (steric factor) of the rings of AL488 and Trp when they collide. This agrees with observations that the quenching of the oxazine-derived dye MR121 by Trp occurs through PET mediated by ring-ring interactions⁵. Trp and Tyr are also implicated in ultrafast electron transfer with riboflavin in flavoproteins¹⁰.

To measure the feasibility of electron transfer between the species of interest, we performed cyclic voltammetry experiments. We observed a Trp oxidation peak at +0.82V vs. Ag/AgCl and an AL488 reduction peak at -0.68V vs. Ag/AgCl. We determined the free energy of the electron transfer reaction using the Rehm-Weller relation¹¹. There is some uncertainty in these values, because the redox processes of the amino acids are chemically irreversible. However, a reasonable estimate of this effect would be no more than 100mV. We observed that the electron transfer from Trp to AL488 is highly favorable, with a free energy of -1.15eV. We note that approximately half of the total quenching by Trp arises from static mechanisms. We also calculated the static quenching constant K_S to be 15.1M^{-1} . Static quenching often arises when the fluorophore and quencher take part in stacking interactions, thus forming a non-fluorescent complex⁹. This could explain the ubiquitous quenching of Alexa dyes by Trp.

Measurements of quenching by Tyr were limited by the low solubility of Tyr in aqueous solution. From fluorescence intensity measurements, we observed a strong quenching, comparable to Trp (Fig. 1). The Stern-Volmer plots from lifetime and intensity measurements show the same slope (Fig. 2b), implying that the quenching occurs entirely through a dynamic mechanism. Because phenylalanine (Phe) does not quench AL488 fluorescence, we surmise that quenching arises from PET between AL488 and the phenolic OH group on Tyr. Indeed, Tyr is known to be a good electron acceptor that undergoes PET⁸. We observed a reduction peak at -0.50V vs. Ag/AgCl, which gave a favorable free energy of -1.38eV for electron transfer from Tyr to AL488.

We observed that Met quenches AL488 primarily by collisional quenching (Fig. 2c), with a rate of $1.67 \times 10^9 \text{M}^{-1} \text{s}^{-1}$. The fraction of total collisions that results in quenching is 0.195 ± 0.008 . The side chain of Met contains a sulfur atom embedded in a saturated hydrocarbon chain that is known to be susceptible to oxidation. Thus, we expect that quenching originates from PET from the reactive sulfur atom. Met displays an oxidation peak at +0.72V vs. Ag/AgCl. We observed that electron transfer from Met to AL488 is also feasible with a free energy of -1.21eV.

The quenching of AL488 by His has both static and dynamic components (Fig. 2d), with a dynamic quenching rate of $1.37 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$. The fraction of total collisions that result in quenching is 0.14 ± 0.01 . The imidazole group of His is known to undergo both oxidation and reduction reactions, making PET a possible mechanism of collisional quenching. His has first oxidation peaks at -0.45 V and $+0.53 \text{ V}$ vs. Ag/AgCl , with a favorable free energy of $\sim -1.40 \text{ eV}$ for electron transfer to or from AL488. Similar to Trp, static quenching most likely occurs through stacking interactions between the imidazole ring of His and AL488.

Although it is often assumed that fluorophores do not interact with amino acids in the proteins they label, we show in this communication that the fluorescence of Alexa dyes 488, 555 and 594 is, in fact, quenched by interactions with Trp, Tyr, Met and His residues through a combination of static and dynamic quenching mechanisms. Indeed, we observed strong dimming of AL488 when the fluorophore is attached less than 3 residues away from a Tyr residue (data not shown). In light of this finding, the potential effect of intramolecular quenching should be considered in the choice of labeling sites, and in the interpretation of data that involves quantitative measurements of fluorescence intensity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Comparison of quenching as measured by fluorescence intensity across four amino acids of interest. Tryptophan exhibits the strongest quenching effect, while Histidine and Methionine show similar quenching rates. Data for Tyrosine is limited to 2mM of quencher concentration due to its low solubility. The inset shows the Tyrosine data at low concentrations with a line to show the slope relative to that of the other amino acids.

Figure 2.

Stern-Volmer plots of Alexa488 quenched by A) Tryptophan, B) Tyrosine, C) Methionine and D) Histidine. Open squares ($\square$) represent total quenching detected by bulk fluorescence intensity measurements (static and dynamic quenching) while shaded squares ($\blacksquare$) represent quenching detected by fluorescence lifetime measurements (dynamic quenching only). Solid lines represent linear fits to the data, except for the bulk fluorescence data of Tryptophan, where the static quenching component is significant and causes a deviation from linearity. Note that data for Tyrosine is limited to 2mM of quencher concentration due to its low solubility. Dotted lines in A, C and D show the Tyrosine data from B for comparison.